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(54) Title: DETECTION OF FERMENTATION-RELATED MICROORGANISMS

(57) Abstract: Unique DNA sequences characteristic of fermentation-related microorganisms are provided which are useful as primers in PCR-based analysis for identification of fermentation-related microorganisms.

DETECTION OF FERMENTATION-RELATED MICROORGANISMS

Field of the Invention

The present invention relates to assays to detect fermentation-related microorganisms.

Description of the Related Art

In traditional winemaking, the indigenous yeasts ferment the grape must. Most modern winemakers, however, inoculate with a pure culture of a selected yeast strain to ensure a rapid, reliable and predictable fermentation. It is thought that indigenous yeasts are suppressed by the competitive effect of addition of a high-density monoculture, but some evidence suggests that indigenous yeast can still participate in the fermentation. A range of commercial yeasts with different winemaking characteristics is available, and a number of those yeast strains may be used in a single winery. Furthermore, unwanted microorganisms may be present which lead to spoilage. Therefore, there is a need for a rapid, simple and accurate method for identifying microorganisms in starter cultures and fermentations.

Past techniques for detecting and identifying fermentation-related microorganisms, especially yeast, include colony morphology, fermentation performance, sugar fermentation tests, tolerance to various stresses (e.g., ethanol tolerance) phenotypes with functional relevance (e.g., flocculation) nutritional requirements (e.g., oxygen), and resistance and sensitivity levels of cycloheximide. These methods, however, have numerous disadvantages, including lengthy analysis periods, inability to differentiate, e.g., different strains of yeast, and lack of reproducibility.

Recent developments in molecular biology and protein chemistry have provided new methods for identifying microorganisms, including DNA restriction fragment length polymorphisms, protein electrophoresis patterns and chromosome fingerprinting. Such techniques have been used for identifying fermentation-related microorganisms. See, for example, Casey et al., *Journal of the American Society of Brewing Chemists*, 48(3):100-106, 1990; Degre et al., *American Journal of Enology and Viticulture*, 40(4):309-315, 1989; Guillamon et al., *Systematic and Applied Microbiology*,

19:122-132, 1992; Hoeben et al., *Current Genetics*, 10:371-379, 1986; Mozina et al., *Letters in Applied Microbiology*, 24(4):311-315, 1997; Paffetti et al., *Research Microbiology*, 146:587-594, 1995; Panchal et al., *Journal of the Institute of Brewing*, 93:325-327, 1987; Querol et al., *Systematic and Applied Microbiology*, 15:439-446, 1992; Vezinhet et al., *Applied Microbiology and Biotechnology*, 32:568-571, 1990; and Vezinhet et al., *American Journal of Enology and Viticulture*, 43(1):83-86, 1992.

Polymerase chain reaction (PCR)-based techniques have also been used to detect fermentation-related microorganisms. See, for example, DeBarros Lopes et al., *Applied and Environmental Microbiology*, 62(12):4514-4520, 1996; Fell, *Molecular Marine Biology and Biotechnology*, 2(3):174-180, 1993; Fell, *Journal of Industrial Microbiology*, 14(6):475-477, 1995; Ibeas et al., *Applied and Environmental Microbiology*, 62(3):998-1003, 1996; Lavallee et al., *American Journal of Enology and Viticulture*, 45(1):86-91, 1994; Lieckfeldt et al., *Journal of Basic Microbiology*, 33(6):413-425, 1993; and Ness et al., *J. Sci. Food Agric.*, 62:89-94, 1993.

Ribosomal genes are suitable for use as molecular probe targets because of their high copy number. Non-transcribed and transcribed spacer sequences associated with ribosomal genes are usually poorly conserved and, thus, are advantageously used as target sequences for the detection of recent evolutionary divergence. Fungal rRNA genes are organized in units. Each unit encodes mature subunits of 18S, 5.8S, and 28S rRNA. The internal transcribed spacer (ITS) region lies between the 18S and 28S rRNA genes and contains two variable non-coding spacers (referred to as ITS1 and ITS2) and the 5.8S rRNA gene (White et al., 1990; In: *PCR Protocols*; Eds.: Innes et al.; pages 315-322). In addition, the transcriptional units are separated by non-transcribed spacer sequences (NTSs). The ITS and NTS sequences are particularly suitable for the detection of different fungal pathogens.

Kumeda et al. (*Applied and Environmental Microbiology*, 62(8):2947-2952, 1996) describes use of PCR to amplify ribosomal DNA internal transcribed spacers in order to differentiate species of *Aspergillus* Section *Flavi*. The ITSl-5.8S-ITS2 region was amplified using universal primers, and the PCR product analyzed by the principle of single-strand conformation polymorphism. In addition, Gardes et al. (In: *Methods in Molecular Biology, Vol. 50:Species Diagnostics Protocols:*

PCR and Other Nucleic Acid Methods, Ed. J.P. Clapp, Humana Press, Totowa, NJ, (1996) pp. 177-186) describes restriction fragment length polymorphism (RFLP) analysis of fungal ITS regions amplified by PCR.

The PCR amplification of fungal ITS has also been described using other than universal primers. These methods allow for more specificity in identifying classes of fungi, or particular species of fungi. Thus, Gardes and Bruns (*Molecular Ecology*, 2:113-118, 1993) identified ITS primers which allow differentiation of DNA from basidiomycetes against ascomycete DNA. Identification of specific species has been observed using PCR primers directed to unique sequences in the ITS1 and/or ITS2 regions of fungal pathogens. See, for example, Hamelin et al., *Applied and Environmental Microbiology*, 62(11):4026-4031, 1996; Mazzola et al., *Phytopathology*, 86(4):354-360, 1996; O'Gorman et al., *Canadian Journal of Botany*, 72:342-346, 1994; and U.S. Patent No. 5,585,238 to Ligon et al.

Of interest to the present application is the disclosure of PCT International Application US99/04251 based on U.S. Application Serial No. 09/037,990 which relates to oligonucleotide primers for the ITS of ribosomal RNA gene regions of fermentation-related microorganisms such as *Botrytis cinerea*, *Penicillium*, *Brettanomyces/Dekkera*, *Saccharomyces*, *Hanseniaspora/Kloeckera*, *Candida krusei/Issatchenkia orientalis*, *Pichia kluyveri*, *Pichia anomala/Candida pelliculosa*, *Debaryomyces carsonii*, and *Saccharomycodes ludwigii*. Also of interest to the present application is the disclosure of PCT International Application US98/25219 based on U.S. Application Serial No. 08/986,727 which relates to oligonucleotide primers for the ITS of ribosomal RNA gene regions of fungal pathogens such as those that infect grape plants.

The present invention addresses the problem of detecting and identifying fermentation-related microorganisms by PCR-based techniques.

Summary of the Invention

The present invention is directed to the identification of different fermentation-related microorganisms, particularly those involved in the production of wine. The present invention provides DNA sequences which exhibit variability between different fermentation-related microorganisms. In particular, the present invention

identifies regions of DNA sequence located in the internal transcribed spacer (ITS) of the ribosomal RNA gene regions of various fermentation-related microorganisms. Primers derived from the ITS can be used in polymerase chain reaction (PCR)-based and other diagnostic assays to determine the presence or absence of specific fermentation-related microorganisms, including those involved in the production of wine. The primers can also be used as molecular probes to detect the presence of target DNA.

Thus, in one aspect, the present invention provides an isolated double stranded nucleic acid of the full length ITS1 or ITS2 region of a fermentation-related microorganism. More particularly, the DNA sequence is selected from among Sequence ID NOS: 6 to 15 and their complementary sequences.

In another aspect, the present invention provides an oligonucleotide primer for identification of a fermentation-related microorganism, wherein the primer is a divergent portion of the ITS1 or ITS2 region of a fermentation-related microorganism. More particularly, the oligonucleotide primer is selected from among Sequence ID NOS: 16 to 24. Furthermore, the oligonucleotide primers may be selected from among sequences which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24, primers which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24 contiguous with 1 to 15 flanking nucleotide bases in the 5' and/or 3' direction of SEQ ID NOS: 6 to 15, and primers of 10 nucleotide bases or longer which contain at least 5 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24 contiguous with from 1 to 15 flanking nucleotide bases in the 5' and/or 3' direction of SEQ ID NOS: 6 to 15. A pair of the foregoing oligonucleotide primers for use in the amplification-based detection of an ITS of a fermentation-related microorganism is also provided.

In yet another aspect, a method is provided for detection of a fermentation-related microorganism which comprises: (a) obtaining DNA from a fungal culture or colony isolated from a fermentation, or from an organism present in a fermentation beverage; (b) amplifying a part of the ITS of the fermentation-related microorganism using the DNA as a template in a polymerase chain reaction with the aforementioned oligonucleotide primers; and (c) detecting the amplified part of the ITS sequence to determine whether the fermentation-related microorganism is present.

In still another aspect, kits are provided which are useful in detecting fermentation-related microorganisms comprising one or more containers, at least one of which comprise an oligonucleotide primer or a pair of oligonucleotide primers according to the invention.

Brief Description of the Sequences in the Sequence Listing:

SEQ ID NO: 1 DNA sequence for the internal transcribed spacer of *Metschnikowia pulcherrima.*

SEQ ID NO: 2 DNA sequence for the internal transcribed spacer of *Candida stellata.*

SEQ ID NO: 3 DNA sequence for the internal transcribed spacer of *Zygosaccharomyces bailii.*

SEQ ID NO: 4 DNA sequence for the internal transcribed spacer of *Kluyveromyces thermotolerans.*

SEQ ID NO: 5 DNA sequence for the internal transcribed spacer of *Torulaspora delbrueckii.*

SEQ ID NO: 6 DNA sequence for the ITS1 of *Metschnikowia pulcherrima.*

SEQ ID NO: 7 DNA sequence for the ITS2 of *Metschnikowia pulcherrima.*

SEQ ID NO: 8 DNA sequence for the ITS1 of *Candida stellata.*

SEQ ID NO: 9 DNA sequence for the ITS2 of *Candida stellata.*

SEQ ID NO: 10 DNA sequence for the ITS1 of *Zygosaccharomyces bailii.*

SEQ ID NO: 11 DNA sequence for the ITS2 of *Zygosaccharomyces bailii.*

SEQ ID NO: 12 DNA sequence for the ITS1 of *Kluyveromyces thermotolerans.*

SEQ ID NO: 13 DNA sequence for the ITS2 of *Kluyveromyces thermotolerans.*

SEQ ID NO: 14 DNA sequence for the ITS1 of *Torulaspora delbrueckii.*

SEQ ID NO: 15 DNA sequence for the ITS2 of *Torulaspora delbrueckii.*

SEQ ID NO: 16 Oligonucleotide Sequence MXL258.

SEQ ID NO: 17 Oligonucleotide Sequence MXL268.

SEQ ID NO: 18 Oligonucleotide Sequence CsF.

SEQ ID NO: 19 Oligonucleotide Sequence CsR.

SEQ ID NO: 20 Oligonucleotide Sequence QAZ165.

SEQ ID NO: 21 Oligonucleotide Sequence QAZ622.

SEQ ID NO: 22 Oligonucleotide Sequence GSZ450

SEQ ID NO: 23 Oligonucleotide Sequence TdF1

SEQ ID NO: 24 Oligonucleotide Sequence TdR1

SEQ ID NO: 25 Oligonucleotide Sequence ITS5.

SEQ ID NO: 26 Oligonucleotide Sequence ITS4.

Detailed Description Of The Invention

The present invention provides unique DNA sequences which are useful in identifying fermentation-related microorganisms. These unique DNA sequences can be used as primers in PCR-based analysis for the identification of fermentation-related microorganisms, or as molecular probes to detect the presence of DNA from fermentation-related microorganisms. The DNA sequences of the present invention include the internal transcribed spacer (ITS) of the ribosomal RNA gene regions of specific fermentation-related microorganisms, as well as primers that are derived from these regions which are capable of identifying the particular microorganism.

The DNA sequences of the invention are from the ITS of the ribosomal RNA gene region of fermentation-related microorganisms. However, the present invention is not limited to detecting the presence of the microorganisms in fermentation operations, i.e., the invention can be used to detect the presence of such microorganisms from any source. There is variability in the ITS DNA sequences from different microorganisms. The ITS sequences can be aligned and compared. Primers can be designed based on regions within the ITS regions that contain the greatest differences in sequence among the fermentation-related microorganisms. The sequences and primers based on these sequences can be used to identify specific microorganisms.

DNA sequences of particular interest include ITS DNA sequences from *Metschnikowia* sp., especially *Metschnikowia pulcherrima* (anamorph *Candida pulcherrima*); *Zygosaccharomyces* sp., especially *Zygosaccharomyces bailii*; *Kluyveromyces* sp., especially *Kluyveromyces thermotolerans*; *Candida* sp., especially *Candida stellata* and *Torulaspora* sp., especially *Torulaspora delbrueckii* (anamorph *Candida colliculosa*). The ITS DNA sequences, as well as primers of interest, are set

forth in SEQUENCE ID NOS: 1-24. The sequences are useful in PCR-based identification of fermentation-related microorganisms.

Methods for use of the primer sequences of the invention in PCR analysis are well known in the art. For example, see U.S. Patent Nos. 4,683,195; 4,683,202 and 5,585,238, the contents of all of which are hereby incorporated by reference.

The primer sequences of the invention can also be used as molecular probes to detect the presence of target DNA. The T_m for the primers ranges from about 48-58 °C at 50 mM salt. The hybridization temperature is approximately 5-10 °C below the melting temperature. Thus, the primers are hybridized to target DNA typically at a temperature ranging from about 43-55°C. Final wash conditions generally range from about 45-55°C at about 36 mM salt concentration. Specific hybridization as used herein means the use of a final high stringency wash in about 0.2X SSPE (salt concentration of about 36 mM) at a temperature appropriate for the particular primer. lX SSPE contains 10 mM NaH_2PO_4, 180 mM NaCl, and 1 mM EDTA, at pH 7.4.

The ITS DNA sequences of the present invention can be cloned from fermentation-related microorganisms by methods known in the art. In general, the methods for the isolation of DNA from microorganism isolates are known. See, Raeder et al., *Letters in Applied Microbiology,* 2:17-20, 1985; Lee et al., *Fungal Genetics Newsletter,* 35:23-24, 1990; and Lee et al., In: *PCR Protocols: A Guide to Methods and Applications*, Innes et al. (Eds.); pages 282-287, 1990; the contents of all of which are hereby incorporated by reference.

Alternatively, the ITS regions of interest can be identified by PCR amplification. Primers to amplify the entire ITS region can be synthesized according to White et al. (1990; In *PCR Protocols*; Eds.: Innes et al., pages 315-322, the contents of which are hereby incorporated by reference).

The ITS sequences were determined and the sequences were compared to locate divergences which might be useful to test in PCR to distinguish the different fermentation-related microorganisms. The sequences of the ITS regions which were determined are shown as Sequence ID NOS: 1 to 5. The DNA sequences for the ITS1 and ITS2 regions are shown as Sequence ID NOS: 6 to 15. From the identification of divergences, numerous primers were synthesized and tested in PCR-amplification.

Purified microorganism DNA and DNA isolated from fermentation cultures and colonies were used as templates for PCR-amplification. Thus, pairs of diagnostic primers were identified, i.e., those which identified one particular fermentation-related microorganism species. Preferred primer combinations are able to distinguish between the different microorganisms in, for example, fermentation cultures. Primer sequences are set forth in Sequence ID NOS: 16 to 24. Thus, while oligonucleotide primers selected from among Sequence ID NOS: 16 to 24 are preferred, primers may also be used which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24. Additionally, primers may be used which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24 contiguous with 1 to 15 flanking nucleotide bases in the 5' and/or 3' direction of corresponding SEQ ID NOS: 6 to 15, and primers of 10 nucleotide bases or longer which contain at least 5 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24 contiguous with from 1 to 15 flanking nucleotide bases in the 5' and/or 3' direction of corresponding SEQ ID NOS: 6 to 15.

The present invention provides numerous diagnostic primer combinations. The primers of the invention are designed based on sequence differences among the microorganism ITS regions. A minimum of one base pair difference between sequences can permit design of a discriminatory primer. In general, primers should have a theoretical melting temperature between about 55 °C to about 65 °C to achieve good sensitivity, and should be void of significant secondary structure and 3' overlaps between primer combinations. Primers are generally at least about 10 nucleotide bases, more preferably at least about 15 to about 20 nucleotide bases.

The oligonucleotide primers of the present invention are particularly useful in detecting microorganisms involved in fermentations, in particular, microorganisms selected from among *Metschnikowia* sp., especially *Metschnikowia pulcherrima* (anamorph *Candida pulcherrima*); *Zygosaccharomyces* sp., especially *Zygosaccharomyces bailii; Kluyveromyces* sp., especially *Kluyveromyces thermotolerans*; *Candida* sp., especially *Candida stellata* and *Torulaspora* sp., especially *Torulaspora delbrueckii* (anamorph *Candida colliculosa*). However, the primers of the present invention can also be used to detect the presence of the foregoing microorganisms from any source.

The present invention also relates to the preparation of "kits" containing elements for detecting fermentation-related microorganisms. Such a kit may comprise a carrier to receive therein one or more containers, such as tubes or vials. Unlabeled or detectably labeled oligonucleotide primers may be contained in one or more of the containers. The oligonucleotide primers may be present in lyophilized form, or in an appropriate buffer. One or more enzymes or reagents for use in PCR reactions may be contained in one or more of the containers. The enzymes or reagents may be present alone or in admixture, and in lyophilized form or in appropriate buffers. The kit may also contain any other component necessary for carrying out the present invention, such as buffers, extraction agents, enzymes, pipettes, plates, nucleic acids, nucleoside triphosphates, filter paper, gel materials, transfer materials, and autoradiography supplies.

The examples below illustrate typical experimental protocols which can be used in the isolation of ITS sequences, the selection of suitable primer sequences, the testing of primers for selective and diagnostic efficacy, and the use of such primers to detect the presence of a fermentation-related microorganism. Such examples are provided by way of illustration and not by way of limitation.

Example 1

Culture of Yeast and Fungal Isolates

Viable isolates of *Candida stellata*, *Kluyveromyces thermotolerans*, *Metschnikowia pulcherrima*, *Torulaspora delbrueckii*, *Zygosaccharomyces bailii*, *Brettanomyces bruxellensis*, *Candida parapsilosis*, *Candida tropicalis*, *Hanseniaspora uvarum*, *Pichia anomala*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bisporus*, and *Zygosaccharomyces cidri* were obtained from the E & J Gallo Winery, the Gallo of Sonoma Winery, the American Type Culture Collection (ATCC) or the Centraalbureau voor Schimmelcultures, The Netherlands (see Table 1). Yeasts were grown on any of several media of choice.

Example 2

Amplification and Sequencing of the Internal Transcribed Spacer (ITS) Regions

The internal transcribed spacer region was amplified from the different isolates directly from the yeast colony using ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'; SEQ ID NO: 25) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; SEQ ID NO: 26). A sterile pipette tip was used to scrape a small amount of colony off of the plate and deposited into a 200-μl microcentrifuge tube containing 5 μ1 each of GeneAmp® 10X PCR Buffer (PE Applied Biosystems, Foster City, CA; part no. N808-0160), 0.2 mM each of dATP, dCTP, dGTP, and dTTP (GeneAmp® dNTPs; PE Applied Biosystems, Foster City, CA; part no. N808-0007), approximately 25 pmole/μl each of ITS5 and ITS4, and 2.5 Units AmpliTaq® DNA polymerase (PE Applied Biosystems; part no. N808-0160). Reactions were run for 35 cycles of 30 s at 94°C, 40 s at 58°C, and 2 min at 72°C, followed by a final elongation step at 72°C for 10 min, on a Perkin Elmer GeneAmp® PCR System 9700 (PE Applied Biosystems). PCR products were purified using QIAquick® PCR Purification Kits (Qiagen Inc., Santa Clarita, CA) to remove any excess primers, nucleotides, and polymerases. Five microliters of the purified PCR products were run on a 1.2% agarose gel with 5 μ1 of pGEM-3Zf(+) double-stranded DNA Control Template (0.2 g/L, PE Applied Biosystems) to approximate concentrations. All products were sequenced using the primers ITS5 and ITS4 (see sequences above; White et al., 1990; In: *PCR Protocols*; Eds.: Innes et al. pp. 315-322). Sequencing was performed on an PE Applied Biosystems 377 Automated DNA Sequencer® using ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kits® (PE Applied Biosystems; part no. 403044). Cycle sequencing products were run over Centri-Sep® spin columns (Princeton Separations, Inc., Adelphia, NJ) to remove excess primers, dye-labeled terminators, nucleotides, and polymerases before being run on the automated sequencer.

Example 3

Selection of Species-Specific Primers

The ITS sequences of the *Metschnikowia* sp., especially *Metschnikowia pulcherrima* (anamorph *Candida pulcherrima*); *Zygosaccharomyces sp.*, especially *Aygosaccharomycees bailii*; *Kluyveromyces* sp., especially *Kluyveromyces thermotolerans*; *Candida* sp., especially *Candida stellata* and *Torulaspora* sp., especially *Torulaspora delbrueckii* (anamorph *Candida colliculosa*) isolates were aligned and primers were designed using Oligo 5.0 (National Biosciences, Inc., Plymouth, MN) in regions of maximum sequence difference between the target species. (See Table 2)

Example 4

Primer Synthesis

Primers were synthesized on an Applied Biosystems 394 DNA/RNA Synthesizer® using phosphoramidite chemistry.

Example 5

Verification of Primer Specificity to Target Species

Different annealing temperatures were tested to determine the optimal temperature for PCR for individual primers. In cases with multiple species-specific primers, different primer combinations were used to determine the best primer combination and annealing temperature to amplify a single species-specific DNA fragment. Species-specific amplification products were produced from primers designed from the ITS region between the 18S and 28S ribosomal DNA subunits of each yeast species of interest.

Species-specific primers were tested against other yeast species to confirm their failure to amplify targets in those species. Specifically, the *Metschnikowia pulcherrima*-specific primers (SEQ ID NOs: 16 and 17) were tested in various combinations with and without primers ITS5 (SEQ ID NO: 25) and ITS4 (SEQ ID NO:

26) against the following species (*Candida stellata*, *Kluyveromyces thermotolerans*, *Torulaspora delbrueckii*, *Zygosaccharomyces bailii*, *Brettanomyces bruxellensis*, *Candida parapsilosis*, *Candida tropicalis*, *Hanseniaspora uvarum*, *Pichia anomala*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bisporus*, and *Zygosaccharomyces cidri*) and did not amplify targets in those species.

Further, the *Candida stellata*-specific primers (SEQ ID NOs: 18 and 19) were tested in various combinations with and without primers ITS5 (SEQ ID NO: 25) and ITS4 (SEQ ID NO: 26) against the following species (*Kluyveromyces thermotolerans*, *Metschnikowia pulcherrima*, *Torulaspora delbrueckii*, *Zygosaccharomyces bailii*, *Brettanomyces bruxellensis*, *Candida parapsilosis*, *Candida tropicalis*, *Hanseniaspora uvarum*, *Pichia anomala*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bisporus*, and *Zygosaccharomyces cidri*) and did not amplify targets in those species.

Further, the *Zygosaccharomyces bailii*-specific primers (SEQ ID NOs: 20 and 21) were tested in various combinations with and without primers ITS4 (SEQ ID NO: 26) and ITS5 (SEQ ID NO: 25) against the following species (*Candida stellata*, *Kluyveromyces thermotolerans*, *Metschnikowia pulcherrima*, *Torulaspora delbrueckii*, *Brettanomyces bruxellensis*, *Candida parapsilosis*, *Candida tropicalis*, *Hanseniaspora uvarum*, *Pichia anomala*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bisporus*, and *Zygosaccharomyces cidri*) and did not amplify targets in those species.

Further, the *Kluyveromyces thermotolerans*-specific primer (SEQ ID NO:22) was tested with ITS5 (SEQ ID NO: 26) against the following species (*Candida stellata*, *Metschnikowia pulcherrima*, *Torulaspora delbrueckii*, *Zygosaccharomyces bailii*, *Brettanomyces bruxellensis*, *Candida parapsilosis*, *Candida tropicalis*, *Hanseniaspora uvarum*, *Pichia anomala*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bisporus*, and *Zygosaccharomyces cidri*) and did not amplify targets in those species.

Finally, the *Torulaspora delbrueckii*-specific primers (SEQ ID NOs: 23 and 24) were tested in various combinations with and without primers ITS4 (SEQ ID NO: 26) and ITS5 (SEQ ID NO: 24) against the following species (*Candida stellata*, *Kluyveromyces thermotolerans*, *Metschnikowia pulcherrima*, *Zygosaccharomyces bailii*, *Brettanomyces bruxellensis*, *Candida parapsilosis*, *Candida tropicalis*, *Hanseniaspora*

uvarum, *Pichia anomala*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bisporus*, and *Zygosaccharomyces cidri*) and did not amplify targets in those species.

Example 6

Utilization of ITS sequences as diagnostic probes to hybridize with target DNA

1. Put chosen concentration of DNA sample in 100 ul of TE, pH 7.0.

2. Add 0.1 volume of 3.0 M NaOH, vortex to mix and incubate at 65°C for 20 min to destroy the RNA and denature the DNA.

3. Spin down condensation. Allow samples to cool to room temp. Neutralize by adding 1.0 volume [110 μ1] of 2M ammonium acetate, pH 7.0, vortex to mix. Spin down to remove solution off of cap. Refrigerate until slot blot apparatus is ready.

4. Apply to 220 μ1 slot-blot apparatus according to manufacturer's protocol.

5. Label ITS sequence probe according to kit manufacturer's recommendation.

6. Prehybridize blots in 1.0% BSA, 1mM EDTA, 0.5 M $NaHPO_4$, pH 7.2, 7.0% sodium dodecyl sulfate for a minimum of 2 hr prior to adding the probe, and then hybridize for 16 hr at 45°C. Initial washes consist of two 30-min washes in 1X SSPE/0.1% SDS at 50°C. Transfer blots to a plastic tray and wash in 1X SSPE for 1 hr, at 50°C with shaking. The final wash should consist of 15 min at 50°C in 0.2X SSPE.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

Table 1

Sources of test isolates

Species name	ID number	Source
Candida stellata	CBS157	CBS[1]
Candida stellata	CBS2649	CBS
Candida stellata	GS056	Gallo of Sonoma[2]
Candida stellata	GS111	Gallo of Sonoma
Candida stellata	GS128	Gallo of Sonoma
Candida stellata	GS174	Gallo of Sonoma
Kluyveromyces thermotolerans	GS003	Gallo of Sonoma
Kluyveromyces thermotolerans	GS104	Gallo of Sonoma
Kluyveromyces thermotolerans	GS119	Gallo of Sonoma
Kluyveromyces thermotolerans	GS127	Gallo of Sonoma
Kluyveromyces thermotolerans	GS132	Gallo of Sonoma
Kluyveromyces thermotolerans	GS137	Gallo of Sonoma
Kluyveromyces thermotolerans	GS138	Gallo of Sonoma
Kluyveromyces thermotolerans	GS166	Gallo of Sonoma
Metschnikowia pulcherrima	GS002	Gallo of Sonoma
Metschnikowia pulcherrima	GS011	Gallo of Sonoma
Metschnikowia pulcherrima	GS018	Gallo of Sonoma
Metschnikowia pulcherrima	GS024	Gallo of Sonoma
Metschnikowia pulcherrima	GS030	Gallo of Sonoma
Metschnikowia pulcherrima	GS043	Gallo of Sonoma
Metschnikowia pulcherrima	GS055	Gallo of Sonoma
Metschnikowia pulcherrima	GS066	Gallo of Sonoma
Torulaspora delbrueckii	GS038	Gallo of Sonoma
Torulaspora delbrueckii	NS1-3	Gallo of Sonoma

Table 1

Sources of test isolates

Species name	ID number	Source
Torulaspora delbrueckii	NS1-5	Gallo of Sonoma
Torulaspora delbrueckii	NS1-9	Gallo of Sonoma
Torulaspora delbrueckii	NS1-11	Gallo of Sonoma
Torulaspora delbrueckii	NS1-16	Gallo of Sonoma
Torulaspora delbrueckii	NS1-19	Gallo of Sonoma
Zygosaccharomyces bailii	QA17	E & J Gallo Winery[3]
Zygosaccharomyces bailii	QA30	E & J Gallo Winery
Zygosaccharomyces bailii	QA31	E & J Gallo Winery
Zygosaccharomyces bailii	QA48	E & J Gallo Winery
Zygosaccharomyces bailii	QA57	E & J Gallo Winery
Brettanomyces bruxellensis	Y153	E & J Gallo Winery
Candida parapsilosis	QA45	E & J Gallo Winery
Candida tropicalis	QA44	E & J Gallo Winery
Hanseniaspora uvarum	GS014	Gallo of Sonoma
Pinchia anomala	34080	ATCC[4]
Saccharomyces cerevisiae	GS084	Gallo of Sonoma
Zygosaccharomyces bisporus	Y476	Gallo of Sonoma
Zygosaccharomyces cidri	36238	ATCC

[1] Centraalbureau voor Schimmelcultures, The Netherlands

[2] Gallo of Sonoma Winery, Healdsburg, CA, USA

[3] E & J Gallo Winery, Modesto, CA, USA

[4] American Type Culture Collection, Rockville, MD, USA

Table 2

Target Organism	Primer Name	Primer Sequence
Metschnikowia pulcherrima	MXL 258	5'-AAGCAGGACCAAACCGGAGG-3' (SEQ ID NO: 16)
Metschnikowia pulcherrima	MXL 268	5'-TATTAGGCCGAAGCAGGACC-3' (SEQ ID NO: 17)
Candida stellata	CsF	5'-TTTGCCAAAACCACTGTGAACA-3' (SEQ ID NO: 18)
Candida stellata	CsR	5'-TTTAAAGATTGGGCGCCTTTC-3' (SEQ ID NO: 19)
Zygosaccharomyces bailii	QAZ165	5'-TGGGAGGATGGGTTCGTC-3' (SEQ ID NO: 20)
Zygosaccharomyces bailii	QAZ 622	5'-GCTATCACTCACCCAATCTC-3' (SEQ ID NO: 21)
Kluyveromyces thermotolerans	QSZ 450	5'-CCTCAGTCAGCAACAGCC-3' (SEQ ID NO: 22)
Torulaspora delbrueckii	TdF1	5'-CTATATGAATGAAGTTAGAGGACGTCTAAAGAT-3' (SEQ ID NO: 23)
Torulaspora delbrueckii	TdR1	5'-GGAAGCACGCACAAGACGTATC-3' (SEQ ID NO: 24)

We claim:

1. An isolated double stranded nucleic acid selected from the group consisting of SEQ ID NOS: 6 to 15 and their complementary sequences.

2. An isolated nucleic acid which specifically hybridizes with the nucleic acid of claim 1.

3. An oligonucleotide sequence for identification of a fermentation-related microorganism, wherein said sequence is selected from the group consisting of SEQ ID NOS: 16 to 24.

4. An oligonucleotide primer which is a fragment of the sequences according to claim 3, and which specifically hybridizes to the ITSl or ITS2 of *Metschnikowia pulcherrima*; *Zygosaccharomyces bailii*; *Kluyveromyces thermotolerans*; *Candida stellata* and *Torulaspora delbrueckii*.

5. An oligonucleotide primer for identification of a fermentation-related microorganism, wherein said primer is selected from the group consisting of primers which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24, primers which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24 contiguous with 1 to 15 flanking nucleotide bases in the 5' and/or 3' direction of SEQ ID NOS: 6 to 15, and primers of 10 nucleotide bases or longer which contain at least 5 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24 contiguous with from 1 to 15 flanking nucleotide bases in the 5' and/or 3' direction of SEQ ID NOS: 6 to 15.

6. A pair of oligonucleotide primers for use in the amplification-based detection of an internal transcribed spacer sequence of a fermentation-related microorganism, wherein the primers are selected from the group consisting of primers which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24, primers which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24 contiguous with 1 to 15 flanking nucleotide bases in the 5' and/or 3' direction of SEQ ID NOS: 6 to 15, and primers of 10 nucleotide bases or longer which contain at least 5 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24 contiguous with from 1 to 15 flanking nucleotide bases in the 5' and/or 3' direction of SEQ ID NOS: 6 to 15.

7. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 16 and SEQ ID NO: 17.

8. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 18 and SEQ ID NO: 19.

9. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 20 and SEQ ID NO: 21.

10. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 23 and SEQ ID NO: 24.

11. A method for detection of a fermentation-related microorganism comprising:

(a) obtaining DNA from a fungal culture or colony isolated from a fermentation, or from an organism present in a fermentation beverage;

(b) amplifying a part of the internal transcribed spacer sequence of said fermentation-related microorganism using said DNA as a template in a polymerase chain reaction with a pair of oligonucleotide primers according to claim 6; and

(c) detecting said amplified part of the internal transcribed spacer sequence to determine whether said fermentation-related microorganism is present.

12. The method according to claim 11, wherein said fermentation-related microorganism is selected from the group consisting of *Metschnikowia pulcherrima*; *Zygosaccharomyces bailii*; *Kluyveromyces thermotolerans*; *Candida stellata* and *Torulaspora delbrueckii*.

13. The method according to claim 12, wherein said fermentation culture or fermentation beverage is a wine fermentation culture or wine fermentation beverage.

14. The method according to claim 12, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 16 and SEQ ID NO: 17.

15. The method according to claim 12, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 18 and SEQ ID NO: 19.

16. The method according to claim 12, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 20 and SEQ ID NO: 21.

17. The method according to claim 12, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 23 and SEQ ID NO: 24.

18. A kit comprising a carrier to receive therein one or more containers, at least one of said containers comprising an oligonucleotide primer according to claim 5.

19. A kit comprising a carrier to receive therein one or more containers, at least one of said containers comprising a pair of oligonucleotide primers according to claim 6.

AMENDED CLAIMS

[received by the International Bureau on 27 April 2001 (27.04.01);
original claims 1 and 2 amended; remaining claims unchanged (1 page)]

1. An isolated double stranded nucleic acid which consists of a member from the group consisting of SEQ ID NOS: 6 to 9 and 12 to 13 and its complementary sequence.

2. An isolated nucleic acid which specifically hybridizes with a nucleic acid selected from the group consisting of SEQ ID NOS: 6 to 9 and their complementary sequences.

3. An oligonucleotide sequence for identification of a fermentation-related microorganism, wherein said sequence is selected from the group consisting of SEQ ID NOS: 16 to 24.

4. An oligonucleotide primer which is a fragment of the sequences according to claim 3, and which specifically hybridizes to the ITSl or ITS2 of *Metschnikowia pulcherrima*; *Zygosaccharomyces bailii*; *Kluyveromyces thermotolerans*; *Candida stellata* and *Torulaspora delbrueckii*.

5. An oligonucleotide primer for identification of a fermentation-related microorganism, wherein said primer is selected from the group consisting of primers which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24, primers which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24 contiguous with 1 to 15 flanking nucleotide bases in the 5' and/or 3' direction of SEQ ID NOS: 6 to 15, and primers of 10 nucleotide bases or longer which contain at least 5 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24 contiguous with from 1 to 15 flanking nucleotide bases in the 5' and/or 3' direction of SEQ ID NOS: 6 to 15.

6. A pair of oligonucleotide primers for use in the amplification-based detection of an internal transcribed spacer sequence of a fermentation-related microorganism, wherein the primers are selected from the group consisting of primers which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24, primers which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24 contiguous with 1 to 15 flanking nucleotide bases in the 5' and/or 3' direction of SEQ ID NOS: 6 to 15, and primers of 10 nucleotide bases or longer which contain at least 5 contiguous nucleotide bases from one of SEQ ID NOS: 16 to 24 contiguous with from 1 to 15 flanking nucleotide bases in the 5' and/or 3' direction of SEQ ID NOS: 6 to 15.

7. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 16 and SEQ ID NO: 17.

8. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 18 and SEQ ID NO: 19.

9. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 20 and SEQ ID NO: 21.

10. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 23 and SEQ ID NO: 24.

11. A method for detection of a fermentation-related microorganism comprising:

(a) obtaining DNA from a fungal culture or colony isolated from a fermentation, or from an organism present in a fermentation beverage;

(b) amplifying a part of the internal transcribed spacer sequence of said fermentation-related microorganism using said DNA as a template in a polymerase chain reaction with a pair of oligonucleotide primers according to claim 6; and

(c) detecting said amplified part of the internal transcribed spacer sequence to determine whether said fermentation-related microorganism is present.

12. The method according to claim 11, wherein said fermentation-related microorganism is selected from the group consisting of *Metschnikowia pulcherrima*; *Zygosaccharomyces bailii*; *Kluyveromyces thermotolerans*; *Candida stellata* and *Torulaspora delbrueckii*.

13. The method according to claim 12, wherein said fermentation culture or fermentation beverage is a wine fermentation culture or wine fermentation beverage.

14. The method according to claim 12, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 16 and SEQ ID NO: 17.

15. The method according to claim 12, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 18 and SEQ ID NO: 19.

16. The method according to claim 12, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 20 and SEQ ID NO: 21.

17. The method according to claim 12, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 23 and SEQ ID NO: 24.

18. A kit comprising a carrier to receive therein one or more containers, at least one of said containers comprising an oligonucleotide primer according to claim 5.

19. A kit comprising a carrier to receive therein one or more containers, at least one of said containers comprising a pair of oligonucleotide primers according to claim 6.

SEQUENCE LISTING

<110> E. & J. Gallo Winery

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/01458

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/04; C12Q 1/70
US CL : 536/23.1, 24.32, 24.3; 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 536/23.1, 24.32, 24.3; 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	JAMES et al. "Use of an rRNA Internal Transcribed Spacer Region to distinguish phylogenetically close related species of the general Zygosaccharomyces and Torulaspora" International J. of Systematic Bacteriology. January 1996, Vol 46, No. 1, pages 189-194.	1-2 --- 3-6, 9-13, 16-19
X	GODDARD et al. "Recurrent invasion and extinction of a selfish gene" PNAS, November 1999, Vol 96, No. 24, pages 13880-13885.	2
X --- Y	JAMES et al. Genbank Accession Number AJ224310 or AJ224312, January 1999.	1-2 --- 3-6, 10, 18-19
X --- Y	VAN NUES et al. "Separate structural elements within internal transcribed spacer 1 of Saccharomyces cerevisiae precursor rRNA" Nucleic Acids Research, 1994, Vol 22, No. 6, pages 912-919.	1-2 --- 3-6, 10, 18-19
X --- Y	VAN der SNADE et al. "Functional Analysis of ITS2 of Saccharomyces cerevisiae ribosomal DNA" J. Mol. Biol. 1992, Vol 22, pages 899-910.	1-2 --- 3-4
Y	Stratagene Cataolg 1988, pg 39.	18-19

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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